THE CYTOLOGICAL EFFECTS OF PODOPHYLLIN AND PODOPHYLLOTOXIN ON THE FERTILIZED EGGS OF CHAETOPTERUS[1]

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One of the clues to the mechanism of mitosis is a study of the ways in which it can be blocked; this has been discussed by Wilson (1963). There have been many such studies, utilizing a wide variety of chemical and physical agents; one of the best known of these agents is colchicine, which has been extensively employed by Eigsti and Dustin and their co-workers, and by many others. Colchicine apparently acts by blocking the mitotic division at metaphase, destroying the spindle in the process; chromosome replication is not affected and polyploidy may result. Podophyllin (derived from the mandrake root) is a similar mitotic poison which destroys the spindle (Sullivan and Wechsler, 1947); however, polyploidy has apparently not been reported to be a sequence of its use. Cornman has made extensive investigations into the action of this substance (reviewed by Cornman and Cornman, 1951) and of related or derived compounds, such as podophyllotoxin and quercetin. The early interest in these substances derived from their apparent efficacy against certain forms of warts (*condylomata acuminata*) and, later, against cancerous cells (see Cornman and Cornman, 1951, and Biesele, 1958, 1962, for specific references). The latter application has not proved to be especially valuable, but podophyllin and its derivatives are nevertheless of considerable interest as antimitotic agents, because of their great potency at very low concentrations.

Biesele (1958) has extensively reviewed the literature on the action of mitotic poisons, including podophyllin and its derivatives, and colchicine. He lists the following agents as among the poisoners of metaphase and later stages of mitosis: colchicine and its derivatives, a number of physical agents, many organic compounds, podophyllin and related compounds, certain sulfhydryl reagents, quinones and phenols, antifolic acids, etc. Of these, we elected to test podophyllin and podophyllotoxin (the active principle of crude podophyllin resin), using fertilized eggs of the polychaete annelid, *Chaetopterus*. With this biological material, it was possible to have a large population of relatively uniform cells, all of which were at the metaphase of the first meiotic division (Figs. 1 and 2) and therefore at a susceptible stage for an antimitotic agent of this type. Cornman and Cornman (1951) used the eggs of certain echinoids (*Arbacia, Lytechinus, Tripneustes* and *Echinarachnius*), of the asteroid, *Asterias*, and of the gastropod, *Chromodoris*. In none of these experiments, with the exception of some tests with *Asterias*, were the eggs at the first meiotic metaphase after fertilization when they were treated.

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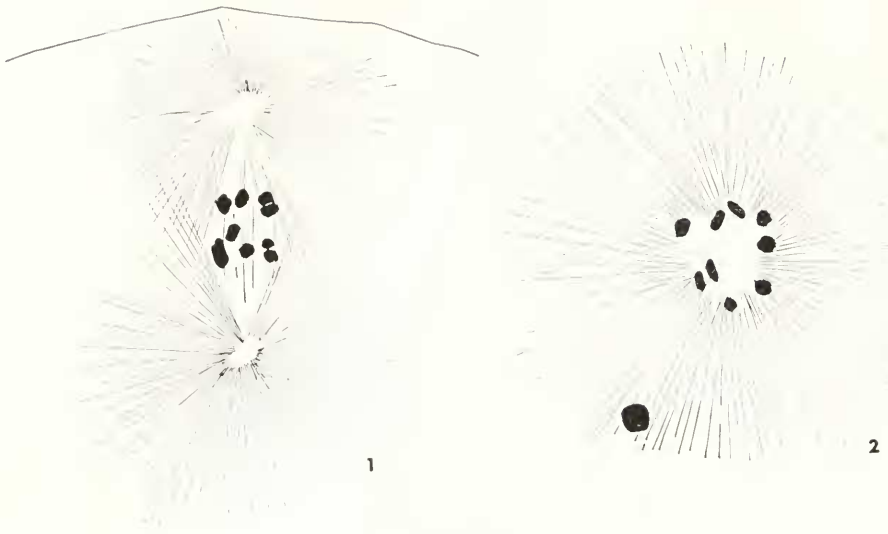


PLATE I

All drawings in this and the succeeding plates are of fixed *Chaetopterus* eggs (whole-mounts, slightly compressed), drawn with the aid of a camera lucida, using 20 × oculars and an oil immersion or a 4-mm. objective, giving total magnifications of 3200 or 1300, respectively. The eggs illustrated were fixed in Kahle's fluid and stained lightly in Harris' acid haematoxylin.

FIGURE 1. Normal first maturation metaphase in *Chaetopterus,* seen in side view. The nine maturation chromosomes are easily countable. Magnification: 1600 ×.

FIGURE 2. Polar view of a normal first maturation metaphase. The aster at only one of the spindle poles is shown. The large black body at the lower left is the nucleolus (which is not shown in Figure 1). Magnification: 1600 ×.

We are indebted to Miss Marion Seiler for her assistance in preparing the final drawings for publication, and to Mr. Donald E. Kent for photography and for his help in other phases of the investigation.

METHODS

Eggs and sperm of *Chaetopterus pergamentaceus* were obtained and inseminated in freshly filtered sea water by the methods outlined by Costello *et al.* (1957). Exactly five minutes after insemination (at which time the egg is at metaphase, preparing to undergo the first meiotic division) measured amounts of podophyllin [2] or podophyllotoxin [2] solution were added to 200 ml. of egg suspension, using a volumetric pipette, while the dish contents were being thoroughly mixed. Both control and experimental dishes were kept on the sea water table; air temperatures varied from approximately 20° C. to 25° C. for different experiments but were relatively constant during the course of any one experiment. At the conclusion of the desired period of treatment, the eggs were washed with large quantities of freshly filtered sea water, and allowed to continue development. Control eggs were cultured in freshly filtered sea water and, except for the absence of the antimitotic

[2] We wish to express our appreciation to Mr. George Motoasca, of S. B. Penick & Company, for samples of these drugs used in the earlier portion of our studies.

agents, were handled in a manner similar to that used for the experimental ova. Treatments were, in most cases, one hour in duration, except where otherwise noted.

Development of the living control and experimental eggs was observed for periods up to three hours, and at intervals after that. Egg-samples were fixed at various times, calculated so that the ova would be killed at the metaphases of first and second polar body formation, and of first and second cleavages. Samples were also fixed approximately 24 hours after insemination, to ascertain what degree of later development, if any, had occurred. Kahle's fixative was routinely used, and the eggs were killed directly on #1 coverslips, by the method described by Henley and Costello (1957). The preparations were stained with Harris' acid haematoxylin, dehydrated in an ethanol series and mounted in damar or Permount. Camera lucida drawings (Figs. 1–17) were made of living and fixed eggs as records; approximately 2100 permanent preparations were studied, utilizing an oil immersion or a 4 mm. objective and 20 × oculars.

The podophyllin and podophyllotoxin stock solutions were made up as follows. Ten mg. of the dry drug were diluted in 10 ml. of distilled water, in a volumetric flask. Appropriate amounts of these stock solutions were then further diluted in volumetric flasks with distilled water to the desired concentrations (0.1 to 0.00001 mg./ml., final concentration, when added to 200 ml. freshly filtered sea water-egg suspension). All solutions were kept refrigerated until shortly before use, when they were allowed to come to room temperature.

Fifty-five series of experiments were carried out.

Results

The sequence of early events in the cytological effects of podophyllin

In fertilized *Chaetopterus* eggs treated with high to moderate levels of podophyllin (0.1 to 0.005 mg./ml.), a characteristic series of early events takes place (Figs. 3–11). In eggs fixed as soon as one minute after the beginning of treatment (with a dosage of 0.005 mg./ml.), there is a suggestion of fading of the asters of the egg maturation figure; the chromosomes look normal in such eggs. At 1½–2 minutes after the beginning of treatment, this process of fading continues (Figs. 3, 9), so that by two minutes after the initiation of treatment, some of the maturation figures appear almost normal, while in others one or both asters are faint or absent, or slightly smaller than normal (Fig. 8). At higher dosages (0.01 mg./ml.), the spindles and asters have disappeared by three minutes after the beginning of treatment (Fig. 5), although the chromosomes remain in essentially the normal first maturation metaphase configuration. By 5 minutes after the beginning of treatment, what appears to be a faint "membrane" begins to appear around the group of chromosomes (Fig. 6), which have now begun to take on a somewhat vesicular appearance, but which still usually remain oriented in a ring of 9 around the periphery of the former spindle area. This same process of "membrane" formation begins about a minute later in eggs treated with lower doses of podophyllin. The containment of the chromosomes within a common membrane (Fig. 7) remains more or less unchanged for a period of a little less than half an hour, after which the chromosome vesicles are released, to lie loose in the cytoplasm (Fig. 10). Cornman and Cornman (1951) described a somewhat similar series of events in

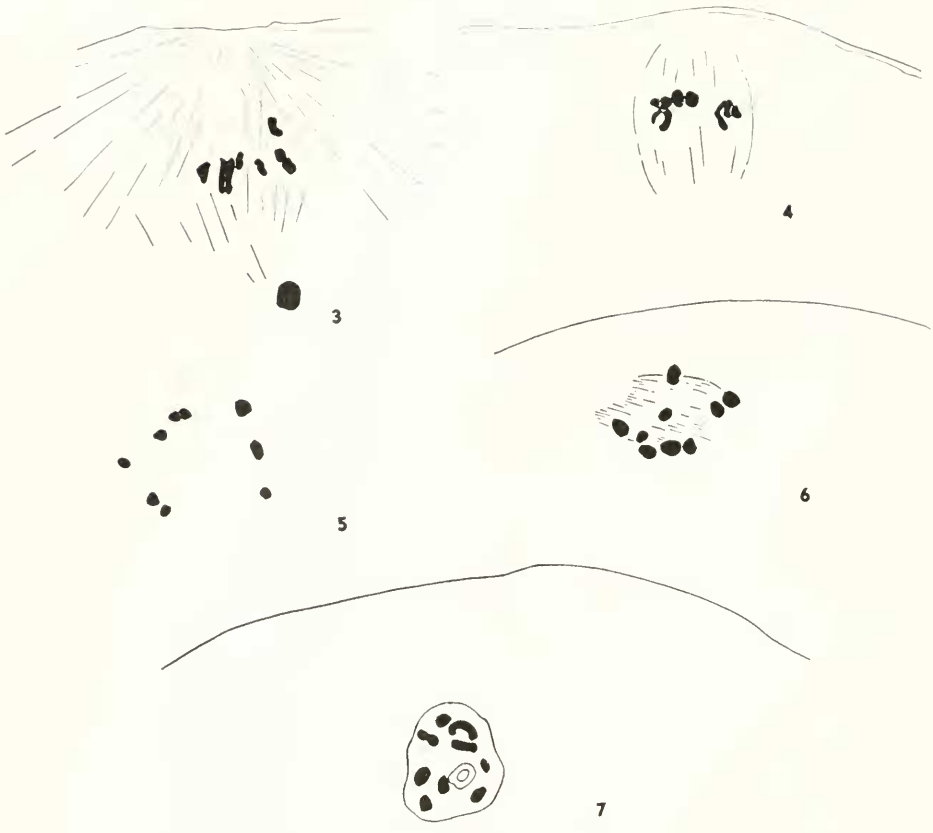


PLATE II

Illustrations in Plate II are of ova from the same experiment, treated with 0.01 mg./ml. podophyllin, beginning five minutes after insemination. Magnification: 1600 ×.

FIGURE 3. One aster (the inner one) has completely disappeared, and part of the first maturation spindle (seen in side view) is likewise no longer visible, in this egg fixed 90 seconds after the initiation of treatment. The large round black body is the nucleolus. Only 8 of the haploid complement of 9 chromosomes could be definitely counted.

FIGURE 4. In this egg, fixed three minutes after the beginning of treatment, both asters have entirely disappeared, and the maturation spindle (side view) is only faintly visible. The fertilization membrane is present at the surface of the egg. (The wrinklings of this membrane, described in the text as typical results of podophyllin treatment, are not evident in this fixed material.) It appeared that only 8 of the 9 chromosomes were present, but it is likely that one of the larger masses of chromatin actually represents two chromosomes.

FIGURE 5. All 9 chromosomes are easily countable, in this polar view of the characteristic first maturation metaphase ring configuration. Both asters and the spindle have completely disappeared, and in the preparation there is only a slight staining at the center of the ring to indicate where the spindle substance was formerly located (not indicated in the drawing). Sample fixed three minutes after the beginning of treatment.

FIGURE 6. In this side view of a first maturation metaphase, the 9 maturation chromosomes are countable, and there is a suggestion (at top) of "membrane" formation. Sample fixed 5 minutes after the beginning of treatment.

FIGURE 7. "Membrane" formation is now completed and the 9 egg maturation chromosomes are entirely enclosed therein, as is the nucleolus (the lighter ring structure). Sample fixed 31 minutes after the beginning of treatment; at this stage, the control eggs were at the metaphase of the first cleavage. Note the similarity in diameter of the chromosome group in this treated egg to that of the normal first maturation metaphase (Fig. 2).

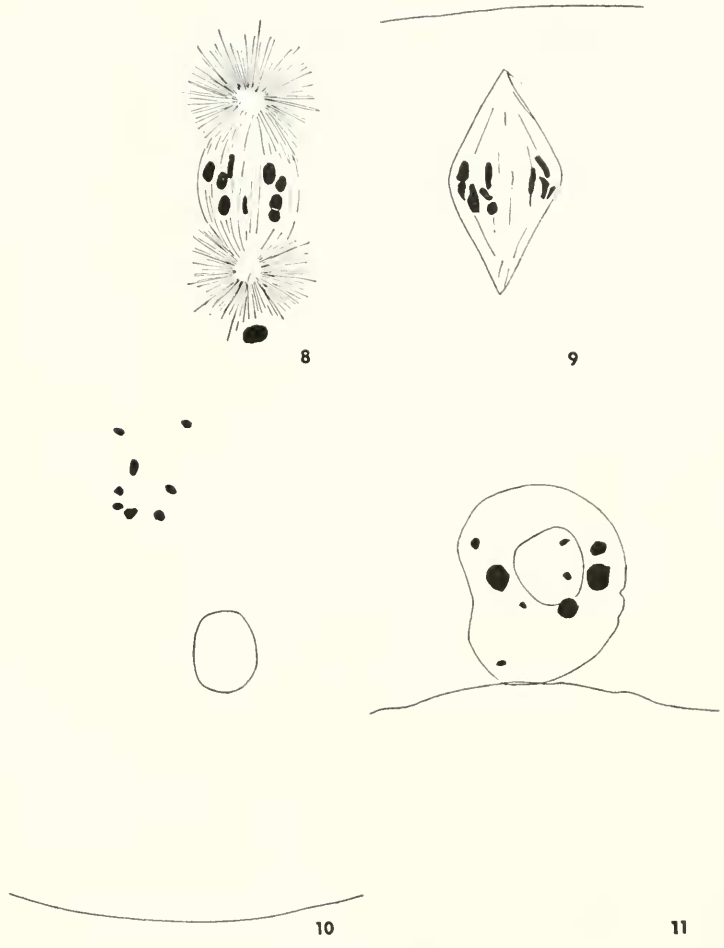


PLATE III

The eggs shown in this plate are from four different experiments, and were treated with varying concentrations of podophyllin (noted for each case); all treatments were begun five minutes after insemination. Magnification: 1600 ×.

FIGURE 8. There is marked reduction in the diameter of the asters and in the length (but, apparently, not of the diameter) of the maturation spindle, in this side view in an egg from a sample fixed three minutes after the beginning of treatment with 0.0055 mg./ml. podophyllin. The nucleolus is visible near the lower aster.

FIGURE 9. Side view of a first maturation metaphase. The spindle is still clearly demarcated but the asters have entirely disappeared. Sample fixed three minutes after the initiation of treatment with 0.01 mg./ml. podophyllin (from the same experiment as the eggs shown in Plate II).

FIGURE 10. The egg maturation chromosomes are now lying free in the egg cytoplasm, with no vestige of asters or spindle. The remnant of the "membrane" which formerly enclosed them (see Figure 7) is lying loose in the cytoplasm nearby. Only 8 of the 9 chromosomes could be counted with certainty. Sample fixed 21 minutes after the beginning of treatment with 0.1 mg./ml. podophyllin.

FIGURE 11. In this egg from a sample fixed 115 minutes after the initiation of treatment with 0.0055 mg./ml. podophyllin, the egg chromosomes have become segregated in a large exovate, simulating a polar body (although very much larger than a normal polar body). There has been some fragmentation and, perhaps, re-consolidation of the chromatin, judging from the variation in size of the chromatin bodies.

living *Asterias* eggs treated with podophyllin at the first maturation division. This process of release occurs first for the egg chromosomes, but somewhat later (approximately an hour after the beginning of treatment with 0.005 mg./ml.) it is also evident in the sperm chromosomes which have also become surrounded by a membrane. (See, also, Cornman and Cornman, 1951.) The two groups of chromosome vesicles often eventually come to be intermingled and indistinguishable from one another. The exact sequence of events here appears to depend upon the entrance point of the sperm, with reference to the maturation figure, inasmuch as the two groups of chromosomes sometimes remain discrete. The empty "membranes" remain visible for a brief period (Fig. 10) but eventually disappear.

Occasionally, there is some fusion of the egg chromosome vesicles with one another, so that fewer but larger vesicles are visible (Fig. 4). Usually, however, nine (the haploid number for *Chaetopterus*) can easily be counted. Cornman (1949) has also reported this apparent fusion of chromosome vesicles in living *Asterias* eggs which had been treated with podophyllin.

The disappearance of the asters and spindles is so complete that there remains no evidence of these structures, except for a slightly more homogeneous staining of the general area they formerly occupied. (By the methods used, there is almost always some staining of the egg cytoplasm with haematoxylin, although this is not sufficiently great to interfere with observations.)

The general sequence of events, as described above, occurs after almost all except the low doses of podophyllin (0.00005–0.00001 mg./ml.), although the tempo is noticeably faster in eggs treated with the higher concentrations. There is no further visible change in the eggs, and no further development occurs at doses between 0.01 mg./ml. and 0.0025 mg./ml. There was no evidence of any conspicuous cyclic growth, disappearance and reappearance of chromosome vesicles, such as were described by Cornman and Cornman (1951) for podophyllin-treated echinoderm eggs. There was no recovery of treated eggs in our experiments, in contrast to the results reported by Inoué (1952) for colchicine-treated *Chaetopterus* ova.

The most striking feature of these results is the remarkable rapidity with which effects are evident, as soon as 60 seconds after the initiation of treatment. This must mean that podophyllin penetrates the *Chaetopterus* egg very rapidly indeed. Swann and Mitchison (1953) found a comparable rapidity of effect of colchicine in causing the achromatic figure to disappear in treated *Psammechinus* eggs, as did Inoué (1952) for colchicine-treated *Chaetopterus* eggs.

The source of the "membrane" which comes to surround the chromosomes is an interesting problem; we are inclined to suspect that it may be derived from the spindle substance, but there is no real evidence for or against this theory except for the suggestion afforded by Figure 6. Gaulden and Carlson (1951) described the formation of a hyaline globule from the spindle substance of colchicine-treated grasshopper neuroblasts, and Kobayashi (1962) reported the complete disappearance of the spindle in demecolcine-treated *Mespilia* eggs, leaving a hyaline zone, which stained poorly with haematoxylin, around the nucleus. In Kobayashi's experiments, there was recovery from the effects of the mitotic poison, and during the early phases of this recovery process, the hyaline material was associated with the reconstituted spindle, spread over the fibrous structures of the mitotic apparatus.

At each of the first three or four divisions after recovery, the hyaline material was distributed to each of the daughter cells, after which it disappeared.

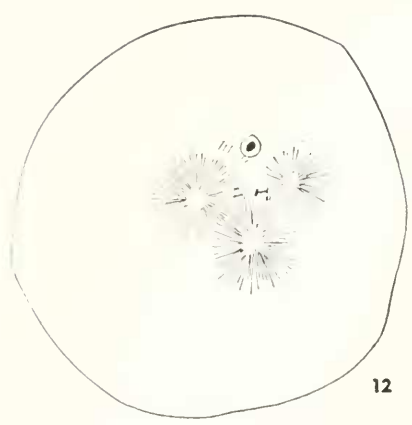
It is interesting that although there is a pronounced reduction in the length of the maturation spindle in podophyllin-treated eggs (compare Figures 1 and 8, for example), the diameter is apparently not affected (compare Figures 2 and 5). (See, also, Inoué, 1952.) Furthermore, the chromosomes usually retain the normal maturation metaphase configuration. This affords confirmation for the idea that the spindle fibers are oriented longitudinally (Inoué, 1952, 1953).

As shown in Figures 3 and 4, the asters were almost invariably the first structures affected by podophyllin, the spindle persisting for as much as one and one-half to two minutes longer. It was observed, also, that the first aster to be affected was usually (but not always—see Figure 3) the one which was nearest the periphery of the egg. It seems reasonable to believe that this is because the antimitotic agent would penetrate through the egg surface and first encounter the more peripheral of the two maturation asters. A similar susceptibility of *Arbacia* egg asters to the effects of another antimitotic agent, colchicine, was reported by Nebel and Ruttle (1938) and by Beams and Evans (1940). Sauaia and Mazia (1961) have demonstrated that in isolated sea urchin egg spindles, previously treated with colcemide (which is very close in chemical structure to colchicine), the asters are likewise the first components of the achromatic figure to be affected. Colcemide applied to the spindles after isolation resulted in no changes, which is not surprising. Beams and Evans (1940) found that the spindle was also destroyed by colchicine, but somewhat later than the asters. They attribute the destruction of asters and spindles to a solation of the cytoplasm, which causes destruction of the spindle, leaving the chromosomes relatively unaffected.

In addition to the rapidity of the effects of podophyllin, another very striking feature is the completeness of the effects, the chromosomes and nucleoli alone remaining more or less unaffected (except for the somewhat vesicular appearance the chromosomes assume). There is no remaining trace of the asters, not even as the "lakes" described by Beams and Evans (1940) as resulting from colchicine-treatment of *Arbacia* eggs. The only evidence of the spindle which persists, as noted above, is a somewhat more homogeneous staining of the egg in the region formerly occupied by this structure. We found no evidence of any fibrous structure in this area; however, its boundary, which we have described as resembling a membrane, is very sharp. It seems quite likely that the association of the chromosomes and the spindle fibers or their remnants may remain in effect somewhat longer than would be apparent at first glance, since the chromosomes retain their characteristic ring-shaped metaphase arrangement for some time after the spindle has visibly disappeared (Fig. 5).

The effects of low doses of podophyllin (0.00005 mg./ml.–0.00001 mg./ml.)

After treatments with concentrations of 0.00005 mg./ml., for periods of 40 to 50 minutes, there was some recovery in treated eggs, although even with these very low doses, the resulting trochophores were usually atypical, with marked ciliary defects and other abnormalities. There was some evidence, also, of fusion of several embryos to form a single "giant." By 24 minutes after the beginning of treatment, there was reduction in the size of the first cleavage asters in some



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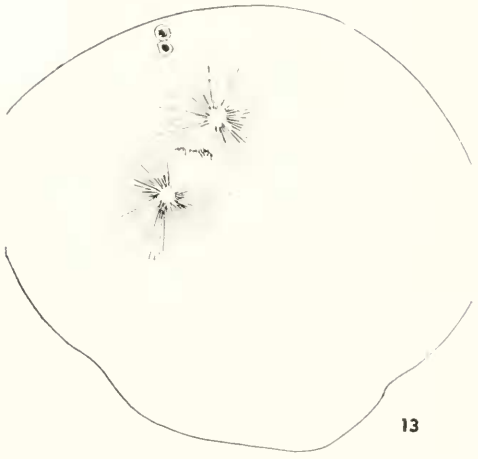

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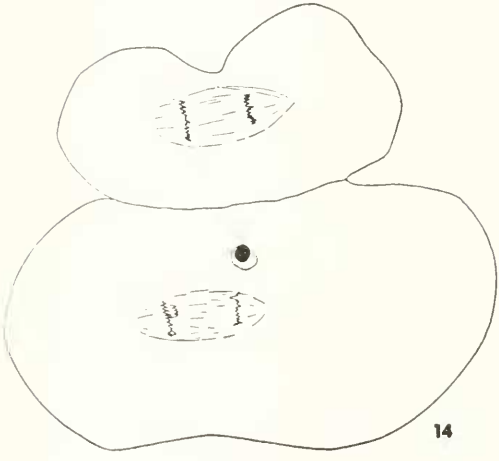

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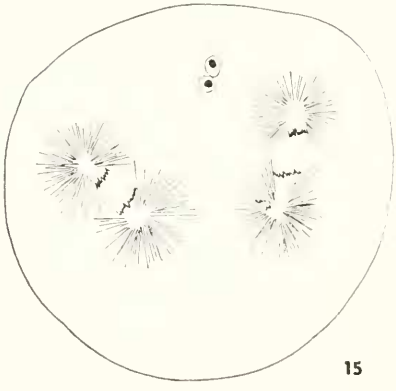

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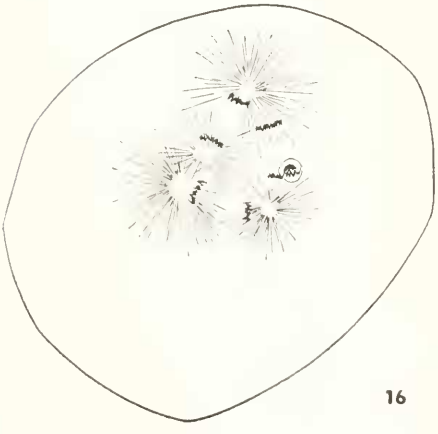

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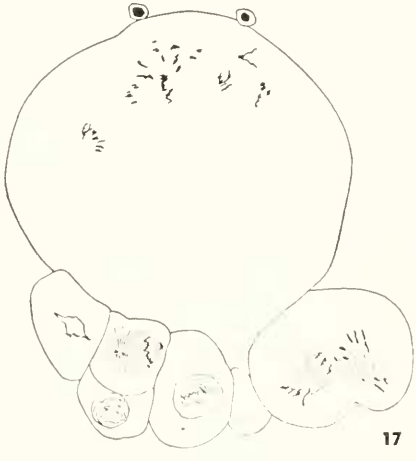

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PLATE IV

eggs, while in others, these structures remained essentially normal. At 47–57 minutes there were some multipolar cleavage figures, and in other eggs, two separate complex metaphases (perhaps male and female in origin?) were apparent. Again, the asters and spindles were fairly normal in a number of ova, but reduced in size in others (see, also, Gaulden and Carlson, 1951, and Kobayashi, 1962). Sixty-eight minutes after the beginning of treatment, much variation was apparent: some eggs had a single large complex metaphase, some had several multipolar figures, some had from one to six interphase nuclei. Usually it appeared that no cytokinesis whatever had taken place, or that it had been partially suppressed, at least. By 113 minutes after the beginning of treatment, the cytological picture was much like that observed at 68 minutes, except that more interphase nuclei (up to 8 in one egg) were observed. Occasionally, cytokinesis had taken place—often resulting in an unbalanced distribution of chromatin, so that one of the daughter "cells" had none.

Treatment with a still lower concentration of podophyllin (0.00001 mg./ml.) (Figs. 12–17) resulted in the appearance of a number of multipolar cleavage figures (Fig. 12) at 27 minutes after the beginning of treatment, and in occasional reduction in size of the asters and spindles (Fig. 13); otherwise, the eggs appeared essentially normal. At 52 minutes after the beginning of treatment, some relatively normal two- (Fig. 14) and four-cell stages, with both polar bodies, were found; other eggs had multiple or multipolar figures in non-divided cytoplasm (Figs. 15,

All the eggs shown in this plate are from the same experiment, treated for 60 minutes with a low concentration of podophyllin (0.00001 mg./ml.), beginning five minutes after insemination. Magnification: 650 ×.

FIGURE 12. Tripolar figure, from an egg-sample fixed 27 minutes after the beginning of treatment. The control eggs fixed at this time were in the prophase to metaphase of the first cleavage, so it seems likely that the mitotic figure shown here is likewise a cleavage spindle, since the development of experimental and control eggs in this series was quite closely synchronous at this point. The chromatin is scanty and thread-like. One polar body is visible at the top of the figure, overlying it on the surface of the egg.

FIGURE 13. In this egg, fixed at the same time as the one shown in Figure 12, the asters are smaller in diameter than usual, but otherwise the figure is similar to the cleavage metaphases in the control eggs. Both polar bodies are present.

FIGURE 14. Two-cell stage, at the anaphase of the second cleavage. The asters are not visible at the ends of the spindle; this second cleavage was synchronous with the same division in the control eggs. One polar body is visible on the larger CD blastomere, below. Fixed 52 minutes after the initiation of treatment.

FIGURE 15. There are two entirely separate anaphase figures in this uncleaved egg fixed at the same time as that shown in Figure 14. No cytokinesis has taken place, but karyokinesis is proceeding, although the two discrete figures are somewhat smaller than normal (compare with Figure 14). Both polar bodies are present (at the top).

FIGURE 16. This complex anaphase configuration is from the same egg-sample as those ova shown in Figures 14 and 15 (fixed 52 minutes after the beginning of treatment). Control samples fixed at this time were proceeding from the two- to the four-cell stage. At least three spindles can be discerned, their relationship to one another being somewhat obscure. One polar body is present beneath the complex array of spindles, at the right.

FIGURE 17. In this egg, fixed 115 minutes after the beginning of treatment (when the controls were in advanced cleavage stages with ±16 cells), abnormal cytokineses has occurred, with six smaller cells in association with one larger one. Two interphase nuclei can be seen in two of the small cells, and most of the mitotic figures appear to be abnormal, including one or more multipolar spindles in the larger cytoplasmic mass. Both polar bodies are present on the periphery of the egg. The larger of the associated cells is undergoing an atypical anaphase.

16). By 115 minutes after the initiation of treatment, there were occasional normal advanced cleavage stages, and other ova with partially or wholly undivided cytoplasm. Quite often, suppression of cytokinesis had occurred only to a degree, although karyokinesis in such cases was usually highly abnormal (Fig. 17). A few multipolar cleavage figures were also observed. The further development of all these eggs was variable; some fairly normal trochophores resulted, while others had marked ciliary defects and there were many dead larvae.

It is important to emphasize here the fact that although there was considerable recovery in the eggs treated with these very low doses, subsequent development was not entirely normal (Figs. 12–17) and the resulting trochophores were almost invariably atypical to at least some degree. Thus, there must be quite profound effects on the egg which are not necessarily apparent at its early stages of development, except in the reduction in size of the spindles and asters.

The multipolar figures observed in cleavage stages of the treated eggs are very similar to those which occur in *Chaetopterus* ova after a number of other experimental treatments, including x-rays (Henley and Costello, 1957) and low temperature (Henley, 1959). They have also been described in the eggs of other forms after a wide variety of experimental treatments, and in rat ascites tumor cells treated with podophyllin (Makino and Tanaka, 1953). Similarly, the suppression of cytokinesis, but not of karyokinesis, also occurs after such treatments.

The fact that the polar bodies usually appeared in a more or less normal fashion (temporally and spatially) after low doses of podophyllin suggests that since the achromatic figures were not usually affected as early after the beginning of treatment with podophyllin at low concentrations as at higher concentrations, polar body formation could proceed in an essentially normal manner. It is of interest that even in eggs treated with higher concentrations of podophyllin (see following section) there was a simulation of polar body formation (Fig. 11); such pseudo-polar bodies were often resorbed, however.

Shape changes in podophyllin-treated eggs; effects on the egg membranes

After treatment with even moderate to high doses of podophyllin, *Chaetopterus* eggs exhibited remarkable simulation of the normal shape changes which characterize the development of this form, even though maturation and cleavage spindles and asters were completely inhibited. With medium concentrations of podophyllin (0.0055 mg./ml., for example), a semblance of polar body formation took place, in which the entire egg nuclear complex was segregated into a large exovate (considerably larger than a normal polar body) (Fig. 11); this exovate usually remained attached to the egg cytoplasm by a stalk. Such "pseudo-polar body formation" occurred at the same time after insemination as the formation of a normal first polar body in the controls. The pseudo-polar body was often resorbed into the egg cytoplasm within about 10 minutes after its initial appearance, and there was usually (but not always) no repetition of the event at the time of second polar body formation in the controls. In occasional ova, exovates occurred in which no chromatin was present, as ascertained by examination of cytological preparations fixed at the time of exovate formation. It is possible that such exovates represent a rupture where the egg cortex and membrane were interrupted at the point of

ovarian attachment. Inoué (1952) has described similar pseudo-polar body formation in colchicine-treated *Chaetopterus* eggs.

Concurrent in time with the characteristic "pear" and polar lobe stages of the control eggs, there were very normal-appearing similar stages in the experimental eggs treated with moderate dosages of podophyllin. The polar lobes were subsequently resorbed by the eggs, approximately 20 minutes after they had appeared, and no further development occurred. Cytological examination of such treated eggs showed that in many cases, at least, the shape changes could be correlated in time with the release of the chromosome vesicles from the membrane. In some instances, abortive cleavages were initiated in the experimental eggs, but except after low doses, such furrows were resorbed within 10–15 minutes.

Similar shape changes in podophyllotoxin-treated eggs, simulating those of normal control eggs, were also observed.

Particularly after treatment with the higher doses of podophyllin (0.1–0.005 mg./ml.) striking effects on the eggs' membranes were observed. Following a dose of 0.1 mg./ml., for example, the membranes wrinkled around the entire peripheries of the eggs, at 57 minutes after the beginning of treatment. Seven minutes later, this wrinkling became very pronounced, and by 157 minutes after the beginning of treatment with this dosage, the membranes became symmetrically exaggerated in their elevation from the surfaces of the eggs. Subsequently, the symmetrical exaggeration often became asymmetrical. The general course of events in this process of exaggerated membrane elevation was strikingly reminiscent of that observed in *Chaetopterus* eggs after cold treatment (Henley, 1959), except that the ova usually did not become denuded of their membranes after podophyllin treatment, as was often the case after cold treatment.

There is a normal series of wrinklings in the *Chaetopterus* egg membrane after fertilization (Pasteels, 1950), but the exaggerated crenations observed after podophyllin treatment are very much more conspicuous.

There is no demonstrable relation between the integrity of the mitotic apparatus and the occurrence of shape changes in the podophyllin-treated *Chaetopterus* egg. In the normal egg of this annelid, the pear-shaped stage coincides with early metaphase of the first cleavage division and the polar lobe stage lasts from approximately the metaphase to anaphase of the same division. Similarly, the appearance of pseudo-polar bodies without chromatin is apparently entirely divorced from the presence of the functional maturation spindle and chromosomes in the egg; this is shown by the fact that in eggs fixed at the time when pseudo-polar bodies were present, the asters and spindles had disappeared (probably about 8–10 minutes earlier judging from the evidence obtained concerning the rapidity of effect of higher concentrations of podophyllin). The ultimate resorption of pseudo-polar bodies and of polar lobes may mean that although the initiation of these morphological changes can occur in the absence of the spindle and asters (but, it should be noted, in the continuing presence of the chromosomes), it cannot continue to the point where polar bodies are actually given off as a consequence of cytokinesis.

The observed exaggerated membrane elevation may result from a separate effect of podophyllin on the cell surface, as distinguished from the effects on asters and spindles.

An attempt to reverse the antimitotic action of podophyllin

The possible antagonistic action of reduced and/or oxidized glutathione was studied, in an attempt to reverse the antimitotic action of podophyllin (see Kawamura, 1960, and Zimmerman, 1960). *Chaetopterus* eggs were subjected to treatment with concentrations of podophyllin (0.012–0.00032 mg./ml.) known to be effective in destroying astral rays; they were then treated, two to five minutes later, with various concentrations of oxidized (0.25–0.025 mg./ml.) or reduced (0.25–0.05 mg./ml.) glutathione. In another series of experiments, the oxidized or reduced glutathione was added to the eggs concurrently with the podophyllin. In no case (regardless of concentration) was there a clear-cut reversal or prevention of injury to the amphiastral system. In some cases (high dosage—0.25 mg./ml.) the effects of oxidized or reduced glutathione plus podophyllin in inhibiting cleavage were greater than those after treatment with podophyllin alone. Such high doses of glutathione, administered to eggs in the absence of podophyllin, were injurious, also.

There is thus no indication that glutathione, either in reduced or oxidized state, can reverse the antimitotic effects of podophyllin, under the conditions of these experiments.

The effects of podophyllotoxin

In general, the effects of podophyllotoxin are entirely comparable to those observed after podophyllin treatment, although the requisite concentrations of podophyllotoxin for a given effect are lower for the active principle than for the crude resin (Table I). A range of concentrations, from 0.01 mg./ml. to 0.00005 mg./ml., inclusive, was tested. In a typical experiment, utilizing podophyllotoxin at a concentration of 0.001 mg./ml., for a duration of approximately 83 minutes, the general cytological picture was like that seen in eggs treated with much higher dosages of podophyllin (0.01 mg./ml., for example); the podophyllotoxin-treated ova showed discrete chromosome vesicles within a membrane, occasional appearance of a large pseudo-polar body, and eventual release of the chromosome vesicles, so that they were lying loose in the egg cytoplasm, by 55 minutes after the beginning of treatment. With a lower concentration of podophyllotoxin (0.00025 mg./ml.), a normal first maturation metaphase was observed in treated eggs as late as 7 minutes after the initiation of treatment, and a normal-appearing second maturation metaphase also appeared at the usual time. By 40 minutes after the beginning of treatment, the first cleavage spindles had disappeared; here it was observed that the spindles were reduced in length and perhaps in diameter. By 65 minutes after the beginning of treatment, only three of 30–40 eggs on a given slide had cleaved and were at a normal-appearing two-cell stage. In the others, no spindles, asters or chromosomes could be seen. Polar bodies were sometimes present on the treated ova, sometimes absent. By 115 minutes after the beginning of treatment, at least one cleavage had occurred in nearly every egg, with most developing ova being at 4-, 6- or 8-cell stages. There was a pronounced delay in cleavage of the treated eggs, as compared with the controls.

After a dose of 0.0005 mg./ml. podophyllotoxin, for a period of approximately one hour, some of the first maturation metaphase asters, spindles and chromosomes looked normal, but in others, there was a range of abnormalities, from a

slight decrease in size of asters and spindles to complete disappearance of these structures. By 27 minutes after the beginning of treament loose chromosome vesicles were present in the cytoplasm of some eggs, and by 53 minutes after the initiation of treatment, this condition held for all the eggs. No further development occurred.

The less concentrated treatment of 0.00005 mg./ml. resulted, at four minutes after the beginning of treatment, in a reduction in size of maturation spindles and asters in certain ova, while in others, these were essentially normal. By the time of the second maturation metaphase there was a slight reduction in size of the

TABLE I

The comparative effects of podophyllin and of podophyllotoxin on the fertilized eggs of Chaetopterus

Conc. and Drug	Effect
0.00001–0.00005 mg./ml. podophyllin	Multipolar figures; abnormal trochophores. Karyokinesis without cytokinesis
0.00005 mg./ml. podophyllotoxin	Reduction in size of asters and spindles; delayed cleavage: 4-cells in exp.; advanced clvgs. in controls
0.0005 mg./ml. podophyllin	Reduction in size of asters and spindles; no polar bodies given off;
0.0005 mg./ml. podophyllotoxin	Very abnormal cleavages at first; some recovery, but not beyond 2-cell stage
0.001 mg./ml. podophyllin	One or two polar bodies present @ 35 abt*; no fusion of pronuclei. Mostly dead by 39 mins. after end of treatment
0.001 mg./ml. podophyllotoxin	Chromosomes in "membrane"; no further development
0.01 mg./ml. podophyllin	Chromosomes within "membrane"; no further develop.
0.01 mg./ml. podophyllotoxin	Chromosomes within "membrane"; no further develop.

All treatments begun 5 minutes after insemination.
* "Abt" = minutes after the beginning of treatment.

spindles and asters. Subsequently, there was considerable delay in development, as compared with the controls, so that in samples fixed 115 minutes after the beginning of treatment, the experimental eggs were in the 4-cell stage, while the controls were in advanced stages of cleavage (about 16 cells). The resulting experimental trochophores were abnormal, with the characteristic atypical features described above as resulting from podophyllin treatment. Thus, at this low concentration, podophyllotoxin has only slight effects on the spindle mechanism, including a decrease in size; a delay in cleavage occurs, and subsequent development is atypical.

The effects of increasing duration of exposure, at the same dosage level, on Chaetopterus eggs treated with podophyllotoxin

To test the effects of increasing the duration of exposure of *Chaetopterus* eggs to podophyllotoxin at a single given concentration, eggs were exposed by the usual techniques to a dosage of 0.0005 mg./ml.; as noted in the preceding section, this treatment could be expected to produce a predictable and reasonably uniform set of results (containment of the chromosomes in a membrane, with subsequent release) when treatment was continued for 60 minutes. In the present series, treatments ranged from 5 to 20 minutes in length.

After the 5-minute treatment (which, like the 10-, 15- and 20-minute treatments, was followed by two washes with large amounts of freshly filtered sea water, to remove the mitotic poison), there was a good deal of recovery, but the eggs were not entirely normal. At the earlier stages after the initiation of treatment, reduction in the size of maturation spindles was noted, as well as some incidence of multipolar figures in the cleavage stages. The 10-minute treatment resulted in considerable variation in effects; these ranged from the presence of thread-like masses of chromatin (sometimes with a suggestion, only, of asters nearby in the eggs fixed 55 minutes after the initiation of treatment, at a stage when the controls were in ±4-cell stages, to fairly normal-looking ±12-cell stages at 175 minutes after the beginning of treatment. In general, it can be said that this treatment resulted in retardation of development and in more marked abnormalities than the 5-minute treatment, but some degree of recovery did occur.

A 15-minute treatment resulted in surface excrescences, of various sizes, simulating cleavage blastomeres, in samples fixed 100 minutes after the beginning of treatment; in some of these pseudo-blastomeres, small abnormal spindles and/or asters were present, in others they were absent. Similar surface excrescences have been reported by Ormsbee *et al.* (1947) for podophyllin-treated mouse tumor cells in tissue culture, by Cornman and Cornman (1951) for podophyllin-treated echinoderm eggs, and by Makino and Cornman (1953) for mouse tissues treated *in vitro* with podophyllotoxin. Thus only a very slight degree of recovery occurred after a treatment of even this short duration, and similar results were observed in eggs treated for 20 minutes. In general, the effects of these shorter exposures to a moderate dilution of podophyllotoxin were comparable to those observed after treatment with lower doses for longer periods.

Five-minute treatments with various concentrations of podophyllotoxin

Three different concentrations of podophyllotoxin (0.001, 0.0003 and 0.0005 mg./ml.) were tested for their effects on *Chaetopterus* eggs after treatments only 5 minutes in duration. At the highest concentration, the cytological picture characteristic of podophyllin and podophyllotoxin treatments was evident at least as soon as 15 minutes after the onset of treatment (10 minutes after the end of treatment); asters and spindles had disappeared and the chromosomes had already taken on a somewhat vesiculated appearance. (The first samples were not fixed until 15 minutes after the beginning of treatment, so we are not able to state how much earlier the characteristic effects were apparent. However, this concentration of podophyllotoxin, in tests over longer periods, resulted in the usual cytological

picture very soon after the initiation of treatment.) No further development occurred. After treatment with 0.0003 mg./ml., maturation was completed normally, and a semblance of cleavage occurred, although it was slightly delayed, as compared with the controls. Other eggs from the same batch, which were left in the experimental solution for as long as 20 minutes as a second type of control (in addition to eggs in plain sea water), showed no signs of recovery, and the cytological effects of podophyllin and podophyllotoxin noted above were seen. With an intermediate concentration (0.0005 mg./ml.) the first indication of an effect was in samples fixed 36 minutes after the beginning of treatment, in the first cleavage, where the spindles and asters were smaller than normal. In samples fixed 60 minutes after the beginning of treatment, there were many uncleaved eggs, some with two fairly normal (but small) spindles, others with a single multipolar spindle. None was observed to have progressed beyond the two-cell stage. However, between that time and 175 minutes after the beginning of treatment, when another sample was fixed, some degree of recovery appeared to have taken place, because there were a good many fairly normal advanced cleavage stages; they developed into swimming forms which exhibited various types of abnormality in ciliation, surface blebs, etc., as is characteristic of trochophores developing from podophyllin-treated eggs. Ova which were left in the podophyllotoxin solution for 175 minutes did not develop farther than the usual stage found after relatively prolonged treatment with these antimitotic agents. It appears, then, that eggs can recover to some extent from a 5-minute treatment with this intermediate concentration of podophyllotoxin, but they are not entirely normal.

Treatment of Chaetopterus eggs with mitomycin C, N-dichloroacetyl DL serine and quercetin

In another series of experiments, the possible antimitotic effects of several other agents were tested on fertilized *Chaetopterus* eggs; these agents included mitomycin C,[3] N-dichloroacetyl DL serine (sodium) and quercetin, the last-named substance being a pigment component of crude podophyllin resin which has been reported by Cornman and Cornman (1951) to retard division of echinoderm eggs, but to have no effect in destroying the achromatic figure. The general methods followed in these experiments were the same as those described above for podophyllin and podophyllotoxin treatments.

Mitomycin C was shown by Merz (1961) to induce chromosome breaks and inhibition of mitosis in root tip chromosomes of *Vicia faba,* and by Matsumoto and Lark (1963) to block DNA synthesis in bacteria. In concentrations of 0.002 mg./ml. or 0.0001 mg./ml. this substance did not affect the division of *Chaetopterus* ova, nor their subsequent development, even when the treatment was continued for an hour or longer.

N-dichloroacetyl DL serine, reported by Levi *et al.* (1960) to cause regression of sarcoma 37 in mice, had no effects, either cytological or developmental, at any of the concentrations tested (1–0.005 mg./ml.), except that there were some ciliary defects in a few of the experimental larvae observed the day following treatment.

Quercetin, even in a concentration as high as 1 mg./ml., resulted in essentially

[3] We are indebted to Dr. Joel Flaks for his assistance in these experiments.

normal trochophores and in no observed cytological abnormalities in samples fixed 5, 32 and 55 minutes, and three hours after the beginning of treatment. There was, however, a slight retardation of cleavage.

Discussion

There are, as Biesele (1958) has pointed out, a number of different places in which interference with one process or another may lead to the production of mitotic abnormalities or complete cessation of cell division. However, the opportunities for obtaining abnormalities as a result of an interference with mitosis in eggs or cleavage blastomeres may be considerably greater than in relatively undifferentiated tissue culture cells.

For normal embryonic development, there has to be a correlation between mitotic events and events such as oöplasmic segregation which lead to differentiation. Within the mitotic events, there is, of course, a coordinated relationship between karyokinesis (chromosomal activities), cytokinesis, and normal centriole replication, plus the axial relations of spindle orientations which must be coordinated with the segregation of cytoplasmic constituents destined to become incorporated into particular cleavage blastomeres. If some mitotic poisons affect the chromosome replication or behavior, others the furrowing, still others centriole replication, and still others cell respiration and metabolism, the possibility for the abnormal distribution of cytoplasmic stuffs becomes so great that the chance of obtaining normal development is practically non-existent.

Cytological effects of podophyllin and podophyllotoxin on other materials

The following table is of interest as a comparison of the effective doses of podophyllin and podophyllotoxin, respectively, required to block cleavage in fertilized marine eggs, as reported in this paper and by Cornman and Cornman (1951):

Chaetopterus	Arbacia**	Echinarachnius**
Podophyllin: 10 mg./L.*	6 mg./L.	0.5–1 mg./L.
Podophyllotoxin: 1.0 mg./L.	0.6 mg./L.	0.02–0.04 mg./L.

* For purposes of comparison, our dosages have here been expressed as mg./L., rather than as mg./ml., as we have done elsewhere in this paper.

** Data of Cornman and Cornman (1951).

It is apparent that the *Chaetopterus* egg is somewhat more resistant to the effects of podophyllin than is the egg of *Arbacia*, and very much more resistant than the egg of *Echinarachnius* (which is notoriously sensitive to any adverse condition, such as a slight increase in temperature). Similarly, a podophyllotoxin concentration of 1.0 mg./L. is required to block *Chaetopterus* eggs, while slightly more than half that amount suffices to block *Arbacia* eggs, and even smaller amounts block *Echinarachnius* eggs. This illustrates again the greater efficacy of the active principle of podophyllin as compared with the crude resin.

MacCardle (1951) found that *in vivo* treatment of mouse sarcoma 37 with N-acetyliodocolchinol methyl ether produced anastral spindles. In living cells (teased preparations), the spindle fibers were not visible, but in Heidenhain iron haematoxylin-stained sections, fragments of fibers were present. There were many multipolar mitoses. Low doses of podophyllin (20 micrograms) produced effects on sarcoma 37 cells similar to those found after acetyliodocolchinol treatment. After microincineration of cells treated with either agent, the spindle area was marked by the presence of large masses of white ash of calcium or magnesium; these were much larger than similar masses present in untreated microincinerated cells. Biesele (1958) points out that this white ash might represent divalent ions which had united with a lipoidal constituent of the spindle liberated by action of the mitotic poisons. He also suggests that if the white ash originated in calcium or magnesium from the chromosomes, this might indicate a damaging effect of the poisons on the chromosomes themselves.

A comparison of the effects of podophyllin with those of colchicine[4]

Inoué (1952), using polarization optics, has studied the effects of colchicine, in concentrations from 1×10^{-5} to 1×10^{-2} *M*, on the first maturation spindle of the *Chaetopterus* egg. He observed that the spindles in such treated eggs began to disappear within a very few moments after the initiation of treatment, and his Plate II shows that this effect is first apparent in a diminution in size of the asters at 3 minutes 15 seconds after the beginning of treatment with 5×10^{-4} *M* (0.2 gm./L.) colchicine. With higher concentrations, the effects are apparent sooner and are complete, with disappearance of the spindle, by 4 minutes 5 seconds after the beginning of treatment with 5×10^{-3} *M*. In general, there was a direct relation between the concentration of colchicine used and the time required for complete disappearance of the spindle.

Soon after the beginning of treatment with colchicine in Inoué's experiments, the characteristic birefringence of the astral rays and continuous fibers of the spindles began to decrease, while the spindle length shortened. As this process of spindle shortening continued, loss of birefringence became more pronounced. The length of the spindles at the time when birefringence disappeared seemed to depend upon the concentration of colchicine used, but in all cases he noted, as did we in the present experiments with podophyllin, that the chromosomes remained oriented on the equatorial plate until the spindle had completely disappeared. He observed that the chromosomes then began to scatter in the egg cytoplasm; this is in contrast to our findings in podophyllin-treated eggs, and he does not describe any process

[4]An important contribution on the mechanism of colchicine inhibition of mitosis by E. W. Taylor (*J. Cell Biol.*, 25: No. 1, Part II, 145–160; 1965) appeared while this paper was in press. H^3-colchicine was shown to have no direct effects on the duration of the cell cycle (of strain K. B. cultured human cells) or on macromolecular (DNA, RNA, and protein) synthesis, at a concentration of colchicine which completely inhibited mitosis. An exposure of 6 to 8 hours at 10^{-7} *M* was sufficient to block essentially all the cells in metaphase, thus indicating that colchicine is bound to the majority of interphase cells. The data are in agreement with the idea of a mechanism involving reversible binding of colchicine to a set of cellular sites, and suggest that if a critical fraction (3% to 5%) of the sites is complexed, the cell is unable to form a functional mitotic spindle. Presumably, a higher concentration of colchicine would be required to disrupt the mitotic spindle than to prevent its assembly.

of containment of the chromosomes within a membrane, such as we found. As the chromosomes began to move inward from the egg periphery in his studies, a characteristic bulge was observed at the periphery of the egg above them; he interprets this as an abortive polar body, presumably comparable to the similar phenomenon described above for podophyllin-treated eggs. With low concentrations of colchicine, Inoué found that the spindle contracted very slowly; the polar regions began to "disintegrate" and as many as seven parallel "spindles" (his quotation marks) were present, each with a pair of chromosomes at its center. We found nothing comparable to this effect in our studies.

The effects of colchicine at the concentrations tested were apparently reversible in Inoué's studies. Eggs treated for 5 and 10 minutes with 10^{-4} *M* mitotic poison were washed in three changes of fresh sea water; at this time the spindle had disappeared and pseudo-polar body formation had taken place. This condition persisted for more than an hour, at which time the polar bulge receded and a small spindle and asters began to appear. In about 3½ hours this spindle had grown to approximately the normal size, and the egg could be successfully inseminated with ensuing polar body formation.

Inoué (1952) interprets his data to indicate that the action of colchicine is to disorganize the orientation of the micelles in astral rays and spindle fibers, most probably by breaking down some chemical bond in or between the micelles and simultaneously causing some of the remaining micelles to contract (as well as breaking down some of the remaining linkages between them). He suggests that colchicine antagonizes the action of some cell component which keeps the spindle substance polymerizing, and which maintains the spindle micelles in their extended form so that they cannot dissociate from one another.

A comparison of the effective concentration of podophyllotoxin (*ca.* 1.0 mg./L.—equal to 2.4×10^{-6} *M*) with the effective dosages of colchicine, used on marine eggs by various authors (see Biesele, 1958, for references), indicates that the former is between 100 and 1000 times as effective in preventing cleavage.

The Merck Index (1960) gives the empirical formula of colchicine as $C_{22}H_{25}NO_6$, with a molecular weight of 399.43. Podophyllin is, of course, a mixture of substances, of which podophyllotoxin is the active antimitotic principle. The formula of podophyllotoxin is given as $C_{22}H_{22}O_8$, with a molecular weight of 414.4. There would, then, be relatively little osmolar difference (less than 4%) between milligram/liter solutions of the two substances, colchicine and podophyllotoxin.

Another study utilizing colchicine was that of Gaulden and Carlson (1951), who described the formation of a hyaline globule in colchicine-treated grasshopper neuroblasts. This globule was seen to arise either from the karyolymph of late prophase or from the spindle of metaphase or anaphase cells. When it originated from the spindle, the first sign of an effect was a reduction in the size of the spindle, to about half its initial size; the globule appeared at one side of the chromosome group and was not surrounded by a membrane. Gaulden and Carlson made three points in summarizing their findings: (1) The greater the concentration of colchicine, the greater its effects in destroying or interfering with development of the spindle. (This is in complete agreement with our findings for podophyllin-treated *Chaetopterus* eggs.) (2) The more completely the spindle was developed at the

time of its exposure to colchicine, the greater the concentration required to destroy it or prevent its further development. (In our experiments, the spindle was in most cases at the first maturation metaphase, so that we are unable to draw any meaningful comparisons on this point.) (3) A series of changes of orientation in the chromosomes was seen to be directly related to changes in the spindle structure. (We have already commented on this finding above—see section on "The sequence of early events in the cytological effects of podophyllin.") Gaulden and Carlson (1951) suggest that colchicine does not destroy the spindle material in grasshopper neuroblasts, but merely alters its molecular configuration, so that it becomes a spherical mass with no mitotic function.

The role of the achromatic figure in mitosis

Hiramoto (1956) reported that in fertilized *Clypeaster* eggs at the dumbbell stage, the mitotic spindle and asters could be completely removed, using a micropipette, and furrowing would continue so that most eggs divided in two. If the spindle were removed in the anaphase or early telophase, division continued in some eggs, while in others the furrow receded. In some cases, the initiation of furrowing was seen after both spindle and asters had been removed as early as the metaphase. The cleavage plane was not affected by the removal of the spindle in Hiramoto's studies, although the speed of furrowing was somewhat slower than normal. He demonstrated, also, that the cleavage plane was unmodified when the position of the mitotic figure was displaced during anaphase or later, by removal of a part of the protoplasm; thus, the cleavage plane is already fixed in the egg cortex.

The effects of colchicine on *Psammechinus* eggs were used by Swann and Mitchison (1953) as a tool to test their hypothesis that the asters are only passive guides for the advancing cleavage furrow. They used the poison to suppress the spindles and asters at a time when the chromosomes had already separated, to see if cleavage would ensue; it did so, even in cases where the asters and spindles had completely disappeared, if the cell had reached mid-anaphase when treatment was initiated. This is in agreement with Hiramoto's findings.

Rappaport (1961) compressed *Echinarachnius* eggs into a torus (doughnut) shape, and found that they then divided only in the spindle region, producing a binucleated horseshoe-shaped cell. The second division following this resulted in the isolation of two uninucleated cells from the ends of the horseshoe. The bend of the horseshoe was binucleated, but within about five minutes after completion of the two "normal" divisions, a furrow appeared between the polar regions of the two asters in the binucleated cell. Thus, a furrow was completed in a region which had never been in close proximity to a spindle or to chromosomes but which was marked by the presence of the two asters. Rappaport suggests, then, that in normal cells, the position of the cleavage furrow may be determined by the "zone of confluence" of the asters.

Kobayashi (1962) has described cases in demecolcine-treated multinucleated *Mespilia* eggs where furrows entered from the egg surface between the asters of neighboring achromatic figures; he states that such cases were not frequent, but were usually encountered when the nuclei were crowded.

Although they did not originate the idea of chemical evocation of cytodieresis,

Cornman and Cornman (1951) have attempted (p. 1479) to explain their results by the assumption that a furrow-organizer is released from the nucleus at the end of prophase and distributed to the equatorial region by the achromatic figure. This furrow-organizer then causes the furrow to form and to progress through the egg. When podophyllin incapacitates the achromatic figure, the furrow-organizer reaches the cortex late, and in an irregular pattern, causing delayed, irregular furrowing. However, the Cornmans (1951, p. 1476) state that furrow activity maintains a relationship with the chromatin and not with the asters. We question this, since furrowing can occur between cytasters in enucleate fragments of marine eggs (Wilson, 1925). We question, also, whether the furrow-organizing substance is released from the nucleus of the mature egg or from the cleavage nuclei at each successive division. It seems more reasonable to assume that the furrow-organizing substance is a cytoplasmic component, possibly derived from the neutral-red-staining granules described by Kojima (1959).

Kojima (1959) has reported that neutral-red-stainable granules appear in the cytoplasm of eggs of three species of Japanese sea urchins (*Hemicentrotus*, *Temnopleurus* and *Mespilia*); at first these granules are uniformly dispersed in the cytoplasm of fertilized ova, but soon they gather around the mitotic figure and are distributed to the two cleavage blastomeres. Subsequently, they appear around the mitotic figures at subsequent cleavages; no change in the number of granules was found. Similarly, in parthenogenetically activated eggs, the granules (at first dispersed in the cytoplasm) gathered around the monaster or cytaster, and in centrifuged eggs in which the granules were segregated, cleavage occurred only in the blastomere containing the granules. If unfertilized *Temnopleurus* or *Mespilia* eggs were centrifuged into two halves, only the centrifugal half, which contains the granules, could develop further after fertilization. Kojima also treated fertilized eggs of these forms before and after vital staining, testing a number of mitotic inhibitors including colchicine and dinitrophenol; he found that especially under the influence of DNP-inhibition, the appearance of the granules after vital staining was inhibited until the eggs were returned to sea water, the granules then gathered around the aster. The effects of colchicine were less clear-cut, there being only a reduction in the observed number of stained granules in such treated eggs; these granules remained dispersed through the cytoplasm. It is important to note here that colchicine and DNP act as inhibitors of mitosis in two different fashions, colchicine being a spindle-destroyer and DNP being a respiratory poison; the significance of Kojima's findings on this point is thus questionable.

Rebhun (1959) has described methylene-blue- and toluidine-blue-stainable granules in the *Spisula* egg, which move to the asters and subsequently migrate in a fashion which suggests the possibility that they are distributed by the mitotic apparatus.

Zimmerman and Marsland (1960) studied *Arbacia* eggs which had been subjected after fertilization to centrifugation at high force (40,000–50,000 *g*) and high pressure (8000–12,000 pounds per square inch) for periods of two to five minutes. They demonstrated that such treated eggs could furrow long before the normal time, often irreversibly, as a consequence of the action of the combination of the two physical agents (if treatment was begun before prophase) or of centrifugation alone (if treatment was begun later). It was necessary that rupture of two groups of

structures have taken place before successful furrowing would occur: (1) the nucleus, and (2) the metachromatic beta granules. Zimmerman and Marsland postulate that the pressure acts by solating the gel structures of the cell, permitting them to break down more readily and facilitating the separation and stratification of the cell's components. They suggest that the furrowing reaction is normally induced by the transport of materials, both nuclear and cytoplasmic in origin, to the two polar regions (spindle poles) of the cell cortex and, perhaps, the mitotic apparatus constitutes the transporting agency. The experimentally-induced reaction, on the other hand, seems to involve only the centripetal pole, and the transport is achieved through the medium of high centrifugal force.

Whatever the source of the furrow-organizer, it is reasonable to assume that in animal cells, there is an accumulation of substance near or in the centrosomal regions, which is transported by the asters to the periphery of the ovum. This streaming would create a fountain movement, of the type described by Spek (1918) for the egg of *Rhabditis*, and by Conklin (1902, 1938) for the egg of *Crepidula*, with streaming from each aster to the cortical equatorial region (equator of the spindle axis) and then back toward the middle of the egg. The furrow as a constricting ring is initiated at the region of convergence of the fountain streaming. With the reduction or destruction of the asters by podophyllin (or by podophyllotoxin), the furrowing (cytokinesis) is inhibited or prevented. Destruction of the spindle prevents separation, etc., of daughter chromosomes (*i.e.*, karyokinesis).

There is nothing, in its molecular structure, to account for the much greater efficacy of podophyllin, as compared with colchicine, but this probably reflects our lack of knowledge of the structure of the mitotic spindle and the astral radiations. We incline toward the view that podophyllin has a direct effect on these structures, and that interference with the passage of some furrow-forming substance through the asters to the furrow region of a dividing cell is a secondary, rather than a primary, effect. The evidence we have presented in this paper supports such a conclusion.

Summary

1. Fertilized eggs of the polychaete annelid, *Chaetopterus pergamentaceus*, were treated with various dilutions of podophyllin and podophyllotoxin, beginning five minutes after insemination. Observations were made on the living eggs and on whole-mount cytological preparations made from samples fixed at various intervals during development. Dosages ranging from 0.1 to 0.00001 mg./ml. were tested.

2. Beginning as soon as 60 seconds after the beginning of treatment with moderate to high dosages (0.1 to 0.005 mg./ml.) of podophyllin, the asters of the egg maturation figure began to fade, followed by disappearance of the spindle by about three minutes after the initiation of treatment. The nine egg chromosomes remained in the ring configuration (characteristic of the first maturation division in this form) for approximately two to five minutes longer (depending on the exact dosage), after which they were gradually enclosed in a membrane-surrounded area which appears to have been derived from the spindle substance. By this time they had taken on a somewhat vesicular appearance. The sperm chromosomes were often similarly affected by the podophyllin, although the onset of these effects was usually somewhat slower than for the eggs. Both egg and sperm chromosomes

remained contained in this membrane for approximately an hour, after which they were released to lie loose in the egg cytoplasm. No further development occurred at these dosages.

3. After treatments with podophyllin concentrations of 0.00005 to 0.00001 mg./ml., a semblance of normal development ensued, although the resulting larvae were usually abnormal. In egg-samples fixed 24 minutes after the beginning of treatment, there was occasional evidence of reduction in size of the asters; by 47–57 minutes after the initiation of treatment, multipolar figures were observed, as well as two entirely separate complex metaphases, often in uncleaved cytoplasm. There was frequently karyokinesis without accompanying cytokinesis. When cleavage continued to occur, it was often retarded and/or abnormal.

4. Shape changes ("pear" and polar lobe stages) characteristic of the normal development of *Chaetopterus* eggs were observed, even after treatment with high dosages which destroyed the achromatic figure. Pseudo-polar bodies, usually without chromatin, were also noted, as well as giant polar bodies.

5. The effects of podophyllotoxin were qualitatively much like those of podophyllin, but the dosage required to produce them was very much lower (0.001 mg./ml. for podophyllotoxin *vs.* 0.01 mg./ml. for podophyllin to completely block division, for example).

6. Even short durations of exposure to either podophyllin or podophyllotoxin (10–20 minutes, as compared with the 60-minute duration routinely used in the other experiments) were sufficient to induce marked abnormality with moderate dosages.

7. The effects of mitomycin C, N-dichloroacetyl DL serine (sodium) and quercetin were also tested. None of these agents was effective in producing cytological abnormality in *Chaetopterus* eggs, at the concentrations tested.

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